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Reversible Immobilization of Antibodies on Magnetic Beads

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A streptavidin-biotin system was utilized to prepare an antibody-polyadenylic acid conjugate which was subsequently attached to commercially available magnetic beads, Dynabeads oligo(dT)₂₅. Biotinylated polyadenylic acid was combined with streptavidin and the resulting polyadenylic acid-streptavidin was conjugated with an antibody-biotin derivative. The immobilized antibody-polyadenylic acid conjugate was separated from the reaction mixture by hybridization with complementary oligonucleotide immobilized on the surface of Dynabeads oligo(dT)₂₅. The immobilized antibody-polyadenylic acid can be released from the carrier, utilizing low-ionic-strength buffers. The system is intended to be utilized in cell sorting, using immobilized antibodies against cell surface antigens. Dissociation of antibody-containing conjugate from magnetic beads is essential for the isolation of viable cells via positive cell sorting. © 1992 Academic Press, Inc.

A number of methods for the immobilization of proteins to solid supports have been described but most involve irreversible immobilization (1,2). Reversible immobilization of proteins through covalent attachment via thioester groups has been reported for coupling to inorganic oxide surfaces, glass, and silica (3). Other methods utilized attachment via disulfide linkages (4) and cleavable thioester bonds (5). Proteins were also immobilized on carbodiimide-activated succinylated glass beads after treatment with 2-mercaptoacetic acid utilizing water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (6). A different type of reversible immobilization of proteins was accomplished by coupling

proteins via arginine residues to a sorbent derivatized with 4-(oxoacetyl)phenoxyacetic acid (7,8). Two systems for reversible immunosorbents are commercially available, the Detachabead system (DynaL, Oslo) and the Flag system (Immunex, Seattle). The former is based on one use of anti-idiotypic antibodies, while the latter employs metal ions requiring antibody-antigen interactions.

The methods above were based on direct covalent coupling of proteins to solid supports. Our concept dealt with immobilization via binding complementary polynucleotides previously attached to both antibody molecules and magnetic beads. To achieve the primary goal, conjugation of polynucleotides with antibodies, several methods of coupling were considered. A hydrazone-based method has been designed for conjugating polynucleotides and proteins (9). Carbohydrate derivatives of polynucleotides prepared from their phosphorimidazolide derivatives (10) have been reported to bind aldehyde groups of oxidized protein molecules in high yield, but we were not able to obtain good yields using this method in our system. Conjugation of antibodies and polynucleotides utilizing water-soluble carbodiimide (11) was also considered, but the random orientation of the coupled polynucleotides and antibodies might sterically block the antigen binding site of the antibody. Other potential methods for the preparation of polynucleotide-antibody conjugates include conjugation of 5'-thiolated oligonucleotides with proteins using 6-maleimidoheptanoic acid succinimidoester (12) or conjugation of polynucleotides with proteins via disulfide bonds (13). Kremsky *et al.* (14) have also described a method for immobilization of polynucleotides containing aldehyde or carboxylic acid groups to hydrazide derivatives.

A multilayered system containing antibody-biotin-streptavidin-biotin-polynucleotide was eventually devised, despite its apparent complexity, since the streptavidin (avidin)-biotin technique has frequently been employed successfully (15,16).

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Polyadenylic acid was biotinylated using biotin-aminocaproyl-hydrazide (17) and glutaraldehyde according to the method of Takahashi *et al.* (18). In contrast to most methods of introduction of biotin into nucleic acids (19), this fast and simple method does not require expensive and sensitive reagents as is the case with enzymatic incorporation of biotin-labeled nucleotides into nucleotide chains (20) or with labeling with photobiotin (21).

Prior to biotinylation IgG was oxidized using sodium periodate. The oxidation generated reactive aldehydes in the carbohydrate part of immunoglobulin molecule without having any effect on the antigen binding function (22,23).

The IgG was then derivatized using biotin-aminocaproyl-hydrazide. This reagent produced stable biotinylated immunoglobulins which retained full immunological activity (24) since binding of biotin-aminocaproyl-hydrazide occurs in the oligosaccharide moiety of the Fc portion of an oxidized antibody molecule, away from the antigen binding site, and does not affect antigen binding capacity. The extended spacer arm of biotin-aminocaproyl-hydrazide reportedly enhances the binding of avidin (streptavidin) to the biotinylated antibody by reducing steric hindrance (17).

Both avidin and streptavidin can serve as a "bridge" connecting biotin derivatives of polyadenylic acid and antibody molecules. Extraordinarily strong noncovalent binding between biotin and avidin (streptavidin) [dissociation constant = 10^{-15} (25)] provides a stable conjugate. However, there may be steric interactions between avidin or streptavidin and a biotinylated antibody molecule, since the biotin binding site of avidin is reported to be 9 Å below the protein surface (26). The possible structural distortion of avidin and antibody molecules can be prevented by using an aminocaproyl spacer.

Streptavidin has the same biotin binding characteristics as avidin but has less nonspecific binding due to its neutral charge and absence of carbohydrate side chains (27). Recently introduced superparamagnetic particles, Dynabeads oligo(dT)₂₅, were chosen as the carrier for the antibody-[biotin-streptavidin-biotin]-polyadenylic acid conjugate. Conditions similar to those used for purification of poly(A) containing mRNA using Dynabeads oligo(dT)₂₅ (28) were adopted for hybridization and dissociation of the conjugate.

MATERIALS AND METHODS

Materials

Streptavidin was purchased from Molecular Probes Inc. (Eugene, OR). Biotin-LC-hydrazide (biotin-aminocaproyl-hydrazide), avidin, and immobilized protein G were purchased from Pierce Chemical Co. (Rockford, IL). ¹²⁵I-labeled goat anti-mouse IgG (sp act 11.9 μCi/

μg; 0.23 μCi/μl) and ¹²⁵I-labeled protein A were obtained from ICN Radiochemicals (Irvine, CA). Dynabeads oligo(dT)₂₅ and magnetic particle concentrator MPC-E 1 were products of Dynal AS (Oslo, Norway) (distributed by Robbins Scientific Corp., Sunnyvale, CA). Oligo nucleotide poly[d(A)]₁₂₋₁₈ [pd(A)₁₂₋₁₈]² was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). Goat IgG, polyadenylic acid (5') [poly(A)], oligo(dT) cellulose, monoclonal anti-protein A, biotin conjugate protein A, Ficoll, glutaraldehyde, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis MO). Centricon 30K miniconcentrators (nominal molecular weight cutoff 30,000) were products of Amicor (Danvers, MA).

Methods

Biotinylation of polyadenylic acid. Freshly prepared solutions of biotin-LC-hydrazide (5 mg/ml H₂O, 2 ml) and 5% glutaraldehyde (1.2 ml) were added to 1 ml of poly(A) solution (2 mg/ml H₂O). The biotin-LC-hydrazide/poly(A) ratio was 5/1 (w/w). After 10 min incubation at 37°C, the resulting poly(A)-biotin was separated by standard ethanol/sodium acetate precipitation (29). The pellet was dissolved in 0.01 M sodium phosphate buffer/1 mM EDTA, pH 7.5 (buffer B). Poly[d(A)]₁₂₋₁₈-biotin was prepared basically in the same way. To initiate precipitation of the oligonucleotide, an ethanolic solution of the polymer was cooled to -70°C and high-speed centrifugation (27,000g × 20 min) was used to collect the resulting precipitate.

Preparation of poly(A)-biotin-streptavidin conjugate. Poly(A)-biotin (1-1.5 mg/ml buffer B) was reacted with streptavidin (4-8 mg/ml in buffer B) at 22°C for 1 h. Different poly(A)-biotin/streptavidin ratios in the reaction mixture were employed (1/3, 1/4, 1/5, 1/10, w/w). An aliquot of each reaction mixture was passed through an oligo(dT)-cellulose column (0.8 × 3.8 cm) equilibrated with 1.0 M NaCl/0.01 M Na phosphate/1 mM EDTA, pH 7.5 (buffer A). After the column was washed with the equilibration buffer, the bound poly(A)-streptavidin was eluted with low-ionic-strength buffer B. Both buffer B eluates and the original reaction mixtures were assayed for A₂₆₀ and A₂₈₀ absorbance.

Poly[d(A)]₁₂₋₁₈-biotin-streptavidin conjugates were prepared in the same way using the optimum poly[d(A)]₁₂₋₁₈-biotin/streptavidin ratio, 1/4 (w/w).

Biotinylation of antibody. Goat IgG [2 mg/ml in 0.1 M sodium acetate/0.02% sodium azide, pH 5.5 (labeling buffer)] was combined with ¹²⁵I-labeled goat IgG to provide labeling at approximately 1,200,000 cpm per milli-

² Abbreviations used: IgG, immunoglobulin G; pd(A)₁₂₋₁₈, oligonucleotide poly[d(A)]₁₂₋₁₈; BSA, bovine serum albumin; IgG-ox, oxidized IgG.

gram of IgG. A small amount of residual free $^{125}\text{I}^-$, which was found in the commercial ^{125}I -IgG, was removed by diafiltration against the labeling buffer. The resulting radiolabeled IgG was oxidized with sodium periodate (solid NaIO_4 added to a final concentration of 20 mM, followed by incubation at 22°C, 30 min). Excess sodium periodate was eliminated by subsequent treatment with ethylene glycol (20 mM final concentration, 10 min). Residual periodate and ethylene glycol were removed by diafiltration against the labeling buffer (0.1 M Na acetate/0.02% NaN_3 , pH 5.5). Oxidized IgG (2 mg/ml labeling buffer) was reacted with biotin-LC-hydrazide [5 mg/ml labeling buffer; IgG-ox/biotin-LC-hydrazide = 1/5 (w/w)] at 22°C for 1 h. The IgG-biotin was separated from excess biotin-LC-hydrazide by ultradiafiltration against buffer B.

Preparation of IgG-poly(A) conjugate: Separation using Dynabeads oligo(dT)₂₅. IgG-biotin (0.5–1.2 mg/ml in buffer B) was reacted with poly(A)-streptavidin (0.3–0.7 mg/ml buffer B) in 1:1 (w/w) ratio for 1 h at 22°C. The resulting IgG-poly(A) conjugate was separated from excess IgG-biotin using direct hybridization with Dynabeads oligo(dT)₂₅. An aliquot (10–25 μl) of reaction mixture containing IgG-poly(A) conjugate was added to 2–10 mg Dynabeads oligo(dT)₂₅ previously equilibrated with binding buffer (1 M LiCl/0.02 M Tris-HCl/2 mM EDTA, pH 7.5). After addition of the binding buffer to a total volume of 0.2 ml, hybridization was allowed to proceed at 22°C for 10 min, during which the suspension was gently mixed several times using a magnet. The beads were separated in MPC-E-1 concentrator and the solution was removed. The beads were then washed repeatedly with 0.2 ml washing buffer (0.15 M LiCl/0.01 M Tris-HCl/1 mM EDTA, pH 7.5) and the supernatants assayed for radioactivity and absorbance A_{260} . The bound material was subsequently released using elution buffer (2 mM EDTA, pH 7.5) at 22 and 37°C. The supernatants were assayed for radioactivity [i.e., ^{125}I -IgG-poly(A)] and absorbance at 260 nm [i.e., for poly(A), as a part of the conjugate] was determined. The total amount of the conjugate and its composition [IgG-poly(A) ratio] were calculated from the results.

In some experiments, Dynabeads oligo(dT)₂₅ were pretreated with 0.5% bovine serum albumin in binding buffer for 10 min. Binding of IgG-poly(A) to the beads was also carried out in the 0.5% BSA/binding buffer.

IgG-poly(dA)_{12–18} conjugates were prepared and separated using the magnetic beads under the same conditions as IgG-poly(A).

The capacity of Dynabeads oligo(dT)₂₅ for the IgG-poly(A) conjugate was compared to the previously determined capacity for free poly(A). Samples of poly(A) were hybridized with the beads, excess unbound poly(A) was washed off, and the adsorbed poly(A) was eluted under the conditions described above. The amount of

eluted, i.e., reversibly bound, poly(A) was calculated from the A_{260} of the elute. For reuse, the beads' residual IgG-poly(A) was removed by treatment with 0.1 M sodium hydroxide followed by reequilibration with binding buffer.

Dissociation of IgG-poly(A) conjugate from magnetic beads in the presence of Ficoll. Ficoll [10% (w/v)] in elution buffer was applied to dissociate the IgG-poly(A) conjugate from the Dynabeads oligo(dT)₂₅ under isotonic cellular conditions. The elution was carried out at 22 and 37°C.

Preparation of anti-protein A-poly(A) conjugate: Hybridization with Dynabeads oligo(dT)₂₅. Biotinylated anti-protein A antibodies (1.8 mg/ml, 20 μl) were combined with poly(A)-streptavidin (0.58 mg/ml in buffer B, 20 μl) and reacted at 22°C for 20 min. An aliquot (10 μl) of the reaction mixture and binding buffer (0.19 ml) was added to 2 mg of Dynabeads oligo(dT)₂₅ (equilibrated with binding buffer). After 10 min the solution was removed using the MPC-E-1 concentrator to separate the beads. After the beads (2 \times) were washed with washing buffer, ^{125}I -labeled protein A (290,000 cpm/ml; 1 mg/ml; 4 μl) was added and the reaction proceeded at 22°C for 5 min. Excess protein A was removed and the protein A-anti-protein A-poly(A) conjugate was subsequently released from the beads by treatment with elution buffer at 22°C (3 \times 0.2 ml, 1 min) and 37°C (2 \times 0.2 ml, 10 min).

RESULTS

Biotinylation of Polyadenylic Acid

Poly(A)-biotin, prepared according to the method of Takahashi *et al.* (18), was used in preparation of IgG-poly(A) conjugates. The biotinylation was easy to execute and isolation of the product by salt/ethanol precipitation was efficient, with a yield of 92–96% of the poly(A) (in A_{260} units).

Preparation of Poly(A)-Biotin-Streptavidin

From the A_{260} and A_{280} analysis of the eluted material it was shown that the resulting poly(A)-biotin-streptavidin conjugate contained poly(A) and streptavidin in a 1:4 (w/w) ratio. If poly(A)-biotin and streptavidin were put in the reaction at this ratio, the entire streptavidin was incorporated in the resulting poly(A)-biotin-streptavidin conjugate.

Biotinylation of Antibody

The antibody-biotin conjugate was prepared as described above. Mild oxidation of immunoglobulin with periodate provided active aldehyde groups in the Fc part of IgG which could subsequently be easily condensed with biotin-LC-hydrazide (17). The modification was

shown not to affect the binding capability of the antibody, which depends mainly on the accessibility of the Fab domain for an antigen.

Note: Prior to oxidation of ^{125}I -labeled IgG it was necessary to remove traces of free $^{125}\text{I}^-$ from the preparation; ultrafiltration was found to be a convenient and reliable method although some minor losses of radioactive material apparently resulted from labeled IgG sticking to the styrene/acrylonitrile ultrafiltration cartridges. Irreversible binding of ^{125}I -IgG to the ultrafiltration membranes was not observed.

Preparation of IgG-Poly(A) Conjugate: Separation Using Dynabeads oligo(dT)₂₅

The IgG-poly(A) conjugate was separated directly with Dynabeads oligo(dT)₂₅ from a reaction mixture of IgG-biotin and poly(A)-streptavidin [the components were in 1:1 (w/w) ratio]. Under the conditions described above, excess IgG-biotin was removed from the beads and the bound IgG-poly(A) conjugate was eluted at 22 and 37°C with low-ionic strength elution buffer (2 mM EDTA). Of the 79–82% of the IgG-poly(A) conjugate that was bound reversibly to the beads, 55–65% IgG-poly(A) was easily eluted at 22°C (two to four portions of elution buffer, 1–2 min incubation) and another 21–24% at 37°C (1 h treatment). About 17–20% of the radioactive IgG-poly(A) remained on the beads after elution; i.e., part of the conjugate was bound irreversibly (see Table 1). This material was removed by subsequent treatment with 0.1 M sodium hydroxide.

The composition of the IgG-poly(A) conjugate was determined from radioactivity and A_{260} analysis; the conjugate consisted of IgG and poly(A) in a 1:3.5–4.1 (w/w) ratio.

The capacity of Dynabeads oligo(dT)₂₅ was 0.69–0.77 μg poly(A) [as a part of the IgG-poly(A) conjugate] per 1 mg beads; this amount was eluted from the beads at 22 and 37°C. The total amount of reversibly bound IgG-poly(A) conjugate was 0.86–0.98 μg per 1 mg beads under conditions described above.

Neither pretreatment of the beads with bovine serum albumin nor the presence of BSA in binding buffer affected the amount of irreversibly bound antibody-poly(A) conjugate, which remained at 16–21% of the material bound.

Washing the beads with 0.1 M sodium hydroxide, which was routinely used as a bead regeneration procedure, did not show any negative effect on subsequent binding efficiency or capacity of the beads.

The IgG-poly(dA)_{12–18} conjugate was also separated from the reaction mixture by direct hybridization with Dynabeads oligo(dT)₂₅. Despite the weaker interactions between shorter polynucleotide sequences, it was not possible to dissociate this conjugate under conditions of higher ionic strength than that required for IgG-

TABLE 1
Separation of IgG-poly(A) conjugate Using Dynabeads Oligo(dT)₂₅

	Total radioactivity ^a (cpm)	Total A_{260} units	Control [IgG-biotin only] ^d (cpm)
Reaction mixture ^b applied to beads	5430 ^b	0.296	5910
Washing buffer elution	3720 ^b	0.066	5900
Total IgG-poly(A) bound to beads	1830 (100%)	n.d.	20
Elution buffer fractions (22°C) ([IgG-poly(A)])	1040 (57%)	0.146	—
Dynabeads after 22°C elution [remaining IgG-poly(A) bound]	750 (41%)	n.d.	10
Elution buffer fractions (37°C) [IgG-poly(A)]	410 (22%)	0.058	—
Dynabeads [residual IgG-poly(A) bound]	370 (20%)	n.d.	10
Total IgG-poly(A) eluted at 22 and 37°C	1450 (79%)	0.204	
IgG:poly(A) (w/w) in combined 22 and 37°C eluates ^c	(1.9 μg IgG) [7.1 μg poly(A)]		
	1:3.7		

Note. Conditions: IgG-biotin (1.2 mg/ml in buffer B) + poly(A)-streptavidin (0.7 mg/ml in buffer B), 1 h at 22°C; reaction mixture (25 μl) + 10 mg Dynabeads oligo(dT)₂₅ [+0.175 ml of binding buffer]—hybridization 10 min at 22°C; washing off excess IgG-biotin (with washing buffer); elution of IgG-poly(A) conjugate (0.2 ml of elution buffer, 1 min at 22°C, then 1 h at 37°C).

^a 1 μg IgG \sim 780 cpm; 1 $A_{260} \sim$ 0.035 mg poly(A).

^b An approximate threefold excess of IgG-biotin in the reaction mixture.

^c The entire 9.0 μg of IgG-poly(A) conjugate was reversibly bound to 10 mg of Dynabeads oligo(dT)₂₅; the capacity was 0.9 μg IgG-poly(A)/mg beads.

^d Nonspecific binding of IgG-biotin to Dynabeads oligo(dT)₂₅ was negligible as shown by the control experiment.

poly(A). Application of the original low-salt elution buffer (2 mM EDTA) was necessary to release the conjugate from the beads.

Dissociation of IgG-Poly(A) Conjugate from Magnetic Beads in the Presence of Ficoll

Presence of the nonionic high-molecular-weight polymer Ficoll [10% (w/v) concentration] in the elution buffer did not affect significantly the dissociation of the IgG-poly(A) complex from Dynabeads oligo(dT)₂₅, although the release of IgG-poly(A) was slower, probably

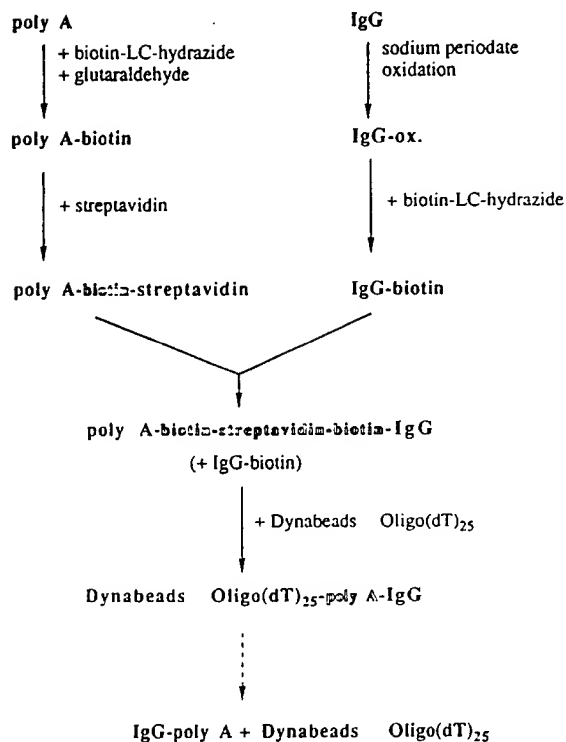


FIG. 1. Schematic depiction of the preparation of IgG-poly(A) conjugate and its hybridization to Dynabeads oligo(dT)₂₅

due to the much higher viscosity of the solution. At 22°C only 46% of radioactivity [¹²⁵I-IgG-poly(A)] was detected in the eluate (compared to 55–65% in eluates without Ficoll) while after treatment at 37°C for 1 h, another 35% of the radioactive IgG-poly(A) was eluted, giving a total yield 81% of IgG-poly(A) reversibly bound to the beads. [The same amount, 79–82% of IgG-poly(A), was released without added Ficoll.]

Preparation of Anti-protein A-Poly(A) Conjugate:

Hybridization with Dynabeads Oligo(dT)₂₅

Approximately 0.5 μg of protein A was bound per 1 mg Dynabeads oligo(dT)₂₅ with the anti-protein A-poly(A) conjugate attached. The experiment was to demonstrate the ability to use this system, antibody-[biotin-streptavidin-biotin]-poly(A) ~ magnetic beads, to isolate biomolecules (Fig. 2).

To demonstrate the reversibility of the binding of complementary nucleotide-labeled biomolecules on oligonucleotide beads, we prepared streptavidin-poly(A) conjugates. These were mixed with a solution containing biotinylated antibody against protein A, forming a streptavidin-labeled antibody complex. This was then mixed with Dynabeads oligo(dT)₂₅, whereupon the antibody was quantitatively removed from the solution.

This was then contacted with ¹²⁵I-labeled protein A, and finally the entire complex was dissociated by elution with a low-ionic-strength buffer with an 80% efficiency, and 0.5 μg of protein A could be bound to 1 mg Dynabeads oligo(dT)₂₅ coupled to the anti-protein-poly(A) conjugate.

DISCUSSION

Biotin-aminocaproyl-hydrazide was used in biotinylation of both antibody (by direct condensation with aldehyde groups in the Fc part of the molecule) and polyadenylic acid [via glutaraldehyde, by the Takahashi method (9)]. The introduction of the aminocaproyl spacer is considered to be very important for the stability of the whole complex with streptavidin, mostly due to reduction of steric hindrance (17). The antibody-polynucleotide conjugate was prepared from IgG-biotin and poly(A)-streptavidin and the product was separated from the reaction mixture via direct hybridization with Dynabeads oligo(dT)₂₅ (Fig. 1). The antibody-polynucleotide conjugate was released from the beads in a good yield; 80% of the IgG-poly(A) bound to the beads was eluted at low ionic strength at 22 and 37°C (approximately 20% of the product was lost due to a kind of irreversible binding to the magnetic carrier). The

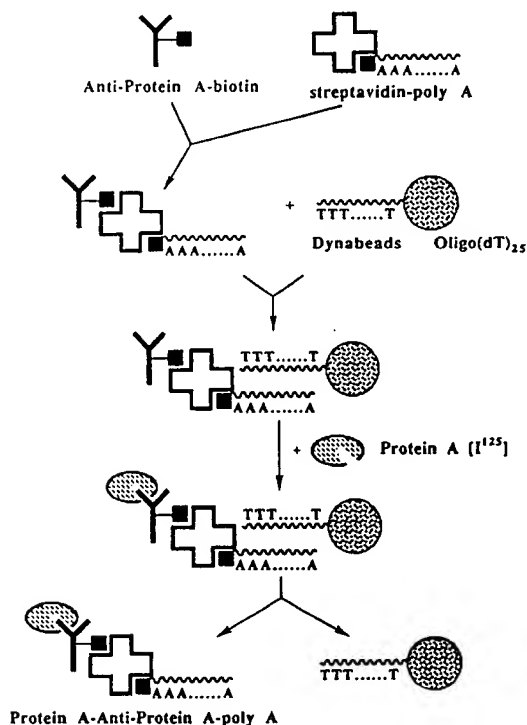


FIG. 2. Schematic depiction of the preparation of anti-protein A-poly(A) conjugate and its hybridization to Dynabeads oligo(dT)₂₅

amount of reversibly bound IgG-poly(A) conjugate seems to be very reasonable compared to other reversible immobilizations which give as low as a 15% yield of released protein (6).

For the isolation of larger biological entities, particularly those present in low concentrations, it would be very useful to minimize the approximately 20% irreversible binding. We tried, therefore, to increase the yield by introducing a oligonucleotide with a shorter chain in the IgG-poly(A) conjugate; however, replacement of poly(A) (ca. 300 nucleotides) by the shorter oligonucleotide, poly[d(A)]₁₂₋₁₈, did not increase the dissociation of the IgG-poly[d(A)]₁₂₋₁₈ conjugate from the magnetic beads and elution could only be completed under low-ionic-strength conditions (2 mM EDTA) with the same yield.

These low salt conditions would not allow isolation of most biological systems, e.g., viable cells, without serious damage to their biological structure and function. Therefore the nonionic polymer, Ficoll, was used to create isoosmotic conditions for gentle separation of viable cells isolated using antibodies attached to a specific antibody-poly(A) ~ magnetic beads complex. Dissociation of the IgG-poly(A) conjugate from the Dynabeads oligo(dT)₂₅ using 10% Ficoll/2 mM EDTA, pH 7.5, was easily accomplished at 22 and 37°C. We conclude that Ficoll or similar oligosaccharide polymers could be employed to create the isoosmotic conditions necessary for isolation of intact eukaryotic cells and other systems.

We have demonstrated the usefulness of the system by preparation of anti-protein A-poly(A) conjugate bound to the magnetic beads; after specific attachment of protein A, the whole complex, protein A-specific anti-protein A antibody-poly(A), was successfully released from the carrier. Isolation of more complex biological systems could be carried out in a similar way.

CONCLUSION

It has been shown that reversible immobilization of antibodies on magnetic beads can be accomplished using polynucleotides bound in a complementary fashion to both the antibody molecules and the beads as a cleavable spacer arm. We used commercially available Dynabeads oligo(dT)₂₅ and the antibody was conjugated with a polynucleotide via streptavidin-biotin linkage. We believe that the reversible binding procedure described here can be used for isolation of different kinds of fragile biological systems such as viruses and viable cells from cell cultures, bone marrow, the peripheral blood stream, or membranes.

One particularly interesting potentiality is the isolation of antigen-specific B cells employing reversibly immobilized antigen.

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